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Folylpoly-γ-glutamate Carboxypeptidase from Pig Jejunum

MOLECULAR CHARACTERIZATION AND RELATION TO GLUTAMATE CARBOXYPEPTIDASE II*

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Charles H. Halsted‡§, Erh-hsin Ling‡, Ruth Luthi-Carter¶, Jesus A. Villanueva‡, John M. Gardner‖, and Joseph T. Coyle¶

From the ‡Department of Internal Medicine, School of Medicine and ‖Center for Engineering of Plants for Resistance against Pathogens, University of California, Davis, California 95616, and the ¶Department of Psychiatry, Harvard Medical School, Boston, Massachusetts 02115

Jejunal folylpoly-γ-glutamate carboxypeptidase hydrolyzes dietary folates prior to their intestinal absorption. The complete folylpoly-γ-glutamate carboxypeptidase cDNA was isolated from a pig jejunal cDNA library using an amplified homologous probe incorporating primer sequences from prostate-specific membrane antigen, a protein capable of folate hydrolysis. The cDNA encodes a 751-amino acid polypeptide homologous to prostate-specific membrane antigen and rat brain *N*-acetylated α-linked acidic dipeptidase. PC3 transfectant membranes exhibited activities of folylpoly-γ-carboxypeptidase and *N*-acetylated α-linked acidic dipeptidase, while immunoblots using monoclonal antibody to native folylpoly-γ-glutamate carboxypeptidase identified a glycoprotein at 120 kDa and a polypeptide at 84 kDa. The kinetics of native folylpoly-γ-carboxypeptidase were expressed in membranes of PC3 cells transfected with either pig folylpoly-γ-carboxypeptidase or human prostate-specific membrane antigen. Folylpoly-γ-carboxypeptidase transcripts were identified at 2.8 kilobase pairs in human and pig jejunum, human and rat brain, and human prostate cancer LNCaP cells. Thus, pig folylpoly-γ-carboxypeptidase, rat *N*-acetylated α-linked acidic dipeptidase, and human prostate-specific membrane antigen appear to represent varied expressions of the same gene in different species and tissues. The discovery of the jejunal folylpoly-γ-carboxypeptidase gene provides a framework for future studies on relationships among these proteins and on the molecular regulation of intestinal folate absorption.

Dietary folates, a heterogeneous mixture of folylpoly-γ-glutamates, are absorbed by a two-stage process of progressive hydrolysis at the jejunal brush border membrane followed by transport of monoglutamyl folate derivatives across the intestinal mucosa (1). Previously, our laboratory (2) purified folylpoly-γ-glutamate carboxypeptidase (FGCP)[2] from human jejunal brush-border membranes as a zinc-activated exopeptidase that releases terminal glutamates sequentially and is stable at pH greater than 8.5. We identified a separate intracellular lysosomal carboxypeptidase in human jejunal mucosa that cleaves folylpoly-γ-glutamates with an endopeptidase mode of action at a pH optimum of 4.5 and that is distinguished from membranous FGCP by its complete inhibition by *p*-hydroxymercuribenzoate (3). Subsequent experiments detected the two separate folate hydrolases in intracellular and brush-border membrane fractions of pig jejunal mucosa, each with properties identical to those found in human jejunum (4). A monoclonal antibody Mab-3 to the purified pig jejunal brush-border FGCP detected a 120-kDa subunit protein that was localized by immunoreactivity to the jejunal brush-border site of *in vivo* hydrolysis of folylpoly-γ-glutamates (5).

Attempts at molecular characterization of pig jejunal FGCP were facilitated by the recent and serendipitous descriptions of the molecular properties of two other proteins, human prostate-specific membrane antigen (PSM) and rat brain *N*-acetylated α-linked acidic dipeptidase (NAALADase). The cDNAs encoding these two proteins demonstrate 67% nucleotide and 65% amino acid sequence identity (6–8) and appear to be homologues of the same enzyme. Previously, we (8, 9) showed that PC3 cells transfected with either of these cDNAs exhibit *N*-acetylaspartylglutamate (NAAG)-hydrolyzing activity characteristic of NAALADase. Others found that PC3 cells transfected with the human PSM cDNA are capable of hydrolysis of folylpoly-γ-glutamate (10) with an exopeptidase activity mechanism similar to that previously described for human jejunal FGCP (2). The discovery that the hydrolysis of both NAAG and folylpoly-γ-glutamate can be attributed to the same molecule (PSM) led to the recommendation that human PSM and rat brain NAALADase be identified under a single IUBMB-approved name (11), subsequently designated glutamate carboxypeptidase II (GCP II; EC 3.4.17.21).

The goals of the present study were to characterize the molecular structure of pig jejunal FGCP while exploring its potential genetic and biological similarities to human PSM and rat NAALADase. We found extensive molecular homology and overlapping catalytic capabilities among pig FGCP, human PSM, and rat NAALADase, consistent with the concept that the three proteins represent varied expressions of the same gene in different species and tissues. The original discovery of the pig FGCP gene provides a molecular framework for future studies on the biological relationships among these proteins and on the integration of jejunal folate hydrolysis within the overall process of the intestinal absorption of dietary folates.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF050502.

§ To whom correspondence should be addressed: TB 156, School of Medicine, University of California, Davis, CA 95616. Tel.: 530-752-6778; Fax: 530-752-3470; E-mail: chhalsted@ucdavis.edu.

[2] The abbreviations used are: FGCP, folylpoly-γ-glutamate carboxypeptidase; NAALADase, *N*-acetylated α-linked acidic dipeptidase; PSM, prostate-specific membrane antigen; NAAG, *N*-acetylated aspartylglutamate; GCP II, glutamate carboxypeptidase II; I100, ileal 100-kDa protein; DPP IV, dipeptidyl peptidase IV; GH, glutamate hydrolase; RFC, reduced folate carrier protein; FBP, folate-binding protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pairs; kb, kilobase pairs.

EXPERIMENTAL PROCEDURES

Reagents—The SuperScript preamplification system was purchased from Life Technologies, Inc. *Taq* DNA polymerase was purchased from Sigma. [α-^{32}P]dCTP (3000 mCi/mmol) and [α-^{35}S]dATP (1000 mCi/mmol) were purchased from Amersham Pharmacia Biotech. A cDNA probe for human actin was obtained from CLONTECH (Palo Alto, CA). *N*-Acetylaspartyl-[3,4-^{3}H]glutamate (42.8 Ci/mmol) and α-[^{32}P]dATP (6000 Ci/mmol) were obtained from NEN Life Science Products. AG 1-X8 anion exchange resin (200–400-mesh, formate form) was purchased from Bio-Rad. 2-(Phosphonomethyl)pentanedioic acid was a gift of Dr. Barbara Slusher, Guilford Pharmaceuticals (Baltimore, MD). Folyl-γ-Glu-γ-[^{14}C]Glu was available as a prior gift from Dr. C. Krumdieck (University of Alabama Birmingham). Purified native pig jejunal FGCP and its monoclonal antibody Mab-3 were available at −70 °C from our previous experiment (5). Peptide-*N*-glycosidase F was purchased from Oxford Glyco Sciences (Bedford, MA). All other reagents were obtained from Sigma, Fisher, and various other commercial sources.

Animal and Human Tissues—Fresh jejunal and ileal mucosal scrapings were obtained from market pigs within 5 min of killing at the University of California (Davis, CA) slaughterhouse and were immediately washed in ice-cold saline, frozen in liquid nitrogen, and stored at −70 °C. They were then used for the preparation of brush-border membranes that were purified ≥ 20-fold according to appropriate marker enzymes and our previously described procedure (5). For subsequent RNA and poly(A$^+$) RNA preparations, portions of pig liver, renal cortex, and duodenal, jejunal, and ileal mucosa were frozen in liquid nitrogen and stored at −70 °C. Human jejunal segments of ~2-cm length were obtained fresh in the operating room from obese patients undergoing elective gastric bypass surgery with gastrojejunal anastomosis, according to acceptable use exemption from the University of California Davis Human Subjects Committee. Segments were opened longitudinally and were washed immediately in ice-cold 4 M guanidium thiocyanate prior to freezing in liquid nitrogen and storage at −70 °C.

Cell Lines—Tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). PC3 cells were grown in MEM supplemented with 2 mM glutamine, 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin; LNCaP cells were cultured in RPMI supplemented with nonessential amino acids, 5% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin. All media reagents were obtained from Life Technologies.

Peptide Microsequencing—As described previously, FGCP was purified from pig jejunal brush-border membranes, and the major subunit protein was identified at 120 kDa by denaturing 6% polyacrylamide gel electrophoresis and immunoblot with Mab-3 monoclonal antibody (5). A parallel gel was stained with Coomassie Blue, and the single 120-kDa band was electroeluted using the Amicon Centrilutor system (12). A peptide digest was prepared by overnight incubation of the eluate with a 50-fold molar excess of cyanogen bromide in 70% formic acid. The resultant peptide fragments were separated on a 7.5% Tricine gel and blotted to ProBlott membranes (Applied Biosystems, Foster City, CA). Peptide sequencing followed the Edman reaction, and amino acids were identified by high performance liquid chromatography (12).

Two peptide sequences contained the sequences KILIARYGKI and LTKELQ, which were 80 and 83% identical to the sequences KIVIARYGKV and LTKELK in the amino acid sequence of human PSM, respectively (6). The corresponding PSM nucleotide sequences encoding these peptides (594–624 and 1428–1446 bp (6)) were used to design sense and antisense oligonucleotide primers for the polymerase chain reaction. Approximately 10 μg of total RNA was extracted from pig jejunal mucosa using TRIzol reagent (Life Technologies) (13), and first-strand cDNA was synthesized using the SuperScript preamplification system (Life Technologies) (14). Following a polymerase chain reaction with the described primers, the amplified product was subcloned into pBluescript II (Stratagene Cloning Systems, La Jolla, CA). A subsequent dideoxy chain termination reaction (15) identified a cDNA sequence of 853 bp that had 87% nucleotide identity to the corresponding region of PSM (6).

Pig Jejunal cDNA Library Construction and Screening—Approximately 10 μg of poly(A$^+$) RNA was prepared from pig jejunal mucosal RNA by the FastTrak 2.0 poly(A$^+$) RNA isolation system (Invitrogen, Carlsbad, CA) (16) and was used for custom construction of a pig jejunal mucosal cDNA library in λZAP by Stratagene Cloning Systems, with a yield of 1.1×10^6 plaque-forming units/ml. The cDNA library was probed with the amplified 853-bp cDNA fragment using established screening methods (17), and positive plaques were purified by secondary and tertiary screening. Following *in vivo* excision and agarose gel electrophoresis, six purified cDNA clones of different sizes between 1.0 and 2.5 kb were identified by Southern analysis using the 853-bp cDNA probe.

cDNA Sequence Analysis—Both strands from each clone were sequenced completely by the dideoxy chain termination reaction using the T3 or T7 polymerase vector primer sequences (15) and by primer walking using gene-specific oligonucleotide primers that were constructed from bases −8 to −5, 203–222, 590–608, 822–836, 948–962, 1233–1252, 1526–1540, 1847–1863, and 2078–2092 (sense) and from bases 284–303, 544–558, 786–801, 1110–1118, 1456–1470, 1645–1660, 1988–2003, and 2237–2245 (antisense). The full cDNA sequence was confirmed independently by cycle sequencing of each clone using the LI-COR 4200 automated sequencer (LI-COR, Lincoln, NE). Clone 7 incorporated all sequences represented in the others, except for an additional 46 bp in the 5′-untranslated region of clone 10 and 25 bp in the 5′-untranslated region of clone 4. No additional sequences were detected in the 5′-untranslated region by rapid amplification of cDNA ends (18). Nucleotide and amino acid sequence identities among pig FGCP, human PSM (6), rat NAALADase (7, 8), and other relevant proteins were analyzed by the BESTFIT and PILEUP programs of version 9.1 of the Genetics Computer Group sequence analysis software package (Madison, WI).

Preparation and Expression of the Cloned Enzyme—A construct of the cDNA of FGCP was prepared by *Hin*dIII and *Xba*I excision from the vector, followed by ligation into the mammalian expression vector pcDNA3 (Invitrogen). One hundred-mm dishes of PC3 cells were transfected with 25 μg of supercoiled plasmid DNA containing the cDNA of pig FGCP or human PSM (construct PSMA2) (9) using the calcium phosphate-mediated method in 50 mM Hepes buffer, pH 7.05 (19). Mock transfected PC3 cells served as controls. Cells were harvested 72 h post-transfection for enzymatic assays by scraping them into 50 mM Tris-HCl buffer (pH 7.4 at 37 °C). Membranes were prepared from the transfected and control PC3 cells by brief sonication followed by centrifugation (35,000 × *g*) for 30 min. The membrane pellets were then solubilized by sonication into 50 mM Tris-HCl plus 0.5% Triton X-100. The protein concentration of the solubilized membrane was determined using the enhanced protocol BCA assay (Pierce) or Bio-Rad kit.

Enzyme Activities—The hydrolysis of NAAG was measured in purified pig jejunal and ileal brush-border membranes and in transfected and mock transfected PC3 cell membranes by radioenzymatic assay, whereby hydrolysis was quantitated via scintillation spectrometry of [^{3}H]glutamate produced from radiolabeled substrate after separation of substrate and product by ion exchange chromatography (20). Assays were initiated by the addition of labeled NAAG at a concentration of 2.5 nM.

Folate hydrolysis was measured in membranes from PSM and FGCP transfectants and mock transfected PC3 cells using substrate folyl-γ-Glu-γ-[^{14}C]Glu and a modification (5) of the method of Krumdieck and Baugh (21) in which terminal [^{14}C]Glu is counted in a liquid scintillation counter after charcoal precipitation of unreacted substrate. Duplicate reactions used 12 μM substrate in 33 mM 3,3-dimethylglutarate buffer containing 0.1 mM zinc acetate. Initial studies evaluated pH dependence and the inhibitory effect of 0.5 mM *p*-hydroxymercuribenzoate in membranes from each cell preparation. Subsequently, kinetic properties were compared in membranes from purified pig jejunal brush borders and from FGCP and PSM transfectants by measurements over a range of substrate concentrations at pH 6.5.

Immunoblots—Membranes from the PC3 cells that were transfected with the cDNA of either human PSM or pig FGCP or that were mock transfected were solubilized in 0.1% Triton X-100. Membrane proteins from the FGCP transfectant were deglycosylated under denaturing conditions using peptide-*N*-glycosidase F according to the manufacturer's protocol. Solubilized membrane proteins and a sample of purified native pig jejunal brush-border FGCP (5) were electrophoresed in parallel on 8% SDS-polyacrylamide gels (22), followed by transfer to polyvinylidene difluoride membranes (Millipore Corp., Marlborough, MA). Protein bands were identified using the monoclonal antibody Mab-3 to the purified native pig FGCP (5) followed by a secondary goat anti-mouse antibody conjugated with alkaline phosphatase (Bio-Rad). The authenticity of Mab-3 immunoreactivity was proven previously by its ability to immunoprecipitate the 120-kDa subunit of FGCP from solubilized pig jejunal brush-border membranes and to localize FGCP in pig intestine immunohistochemically (5).

Northern Blots—Total RNA was extracted from rat brain, LNCaP cells, and pig and human jejunal mucosa (13). Poly(A$^+$) RNA was prepared from pig liver and kidney and duodenal, jejunal, and ileal mucosa (16). Human brain poly(A$^+$) RNA was obtained from CLONTECH Inc. (Palo Alto, CA). A 2.4-kb *Eco*RI–*Nde*I fragment of FGCP was

FIG. 1. **Nucleotide and amino acid sequences of pig FGCP.** Amino acid sequences that correspond with 100 and 83% identity to two peptide sequences from native pig jejunal brush-border FGCP are shown in *boldface type*. There are 146 bp in the 5′-untranslated region, 2255 bp translating to 752 amino acids in the open reading frame, and 186 bp in the 3′-end. The putative transmembrane domain (11) is *underlined*, and the 3′ terminal polyadenylation signal is *double underlined*. Flanking residues Arg^{16} and Arg^{17} are conserved at the N-terminal side of the leucine-rich hydrophobic transmembrane domain between residues 20 and 48. The putative catalytic domain (11) is composed of residues 275–568. There are 12 glycosylation sites (*stars*), of which 10 are conserved in the human PSM sequence (6) and 9 are conserved in the rat NAALADase sequence (7, 8). Zinc-binding residues are conserved at His^{378}, Asp^{387}, Glu^{425}, Asp^{454}, and His^{553} (*closed circles*). Four positively charged residues predicted to be involved in substrate binding are conserved at Arg^{463}, Lys^{503}, Arg^{536}, and Lys^{544} (*open triangles*).

TABLE I

Regional peptide homologies between pig FGCP and selected proteins

The BESTFIT program was used to assess the best regional amino acid sequence similarities and identities among pig FGCP, selected other type II proteins, and other proteins relevant to folate metabolism and membrane transport.

Protein	Reference	GenBank™ accession No.	FGCP region	Similarity	Identity
				%	%
Human PSM	6	M99435	1–750	92	91
Rat NAALADase	7	U28959	1–750	88	83
Rat NAALADase	8	AF040250	1–750	88	83
Human transferrin receptor	26	M32304	9–747	44	32
V. proteolyticus	27	S24834	180–647	43	33
S. griseus	28	S66451	357–888	45	30
Rat 1100	29	AF009921	20–750	50	41
Human DPP IV	30	M80850	259–710	42	29
Human GH	32	U85200	521–706	44	28
Mouse RFC	32	L23785	507–708	38	27
Pig FBP	33	UF9949	4–382	38	29

purified and ^{32}P-labeled for subsequent probing of Northern blots. Rat tissue samples were also probed with a ^{32}P-labeled fragment of human actin cDNA as a positive internal control. After electrophoretic separation in 1.2% agarose, 2.2 M formaldehyde gels and transfer to nylon membranes (Schleicher & Schuell), RNA species were identified by hybridization to cDNA probes as detected autoradiographically (25).

RESULTS

Molecular Sequence of Pig Jejunal FGCP—The complete nucleotide and deduced amino acid sequences of the cDNA of pig FGCP are shown in Fig. 1. The deduced amino acid sequences KILIARYGKIF and MYSLVYNLTKELQ correspond with 100 and 85% identities to two amino acid sequences, KILIARYGKIF and MYILVYGLTKELQ, that were identified in the peptide digest of the native purified enzyme. The complete cDNA of FGCP is composed of 2532 bases: 146 in the 5′-untranslated region, 2253 in the open reading frame that encode 751 amino acids, and 133 in the 3′-untranslated region. The nucleotide and deduced amino acid sequences of pig FGCP were compared with those of human PSM (6) and rat NAALADase (7, 8). Within the open reading frame, the nucleotide identities between pig FGCP and human PSM and rat brain NAALADase were 86 and 83% respectively, while there was very little similarity in the 5′-untranslated region. The amino acid sequence of pig FGCP was 92% similar and 91% identical to that of human PSM and was 87% similar and 83% identical to that of rat NAALADase (Table I). Structural comparisons followed the recent Rawlings and Barrett analysis of human PSM and rat NAALADase (11). The Kyte and Doolittle hydropathy plot (24) of pig jejunal FGCP was identical to those of human PSM and rat NAALADase and typifies a type II protein that conserves a short N-terminal cytoplasmic region and a single hydrophobic transmembrane between residues Tyr^{20} and Ile^{45}. Like human PSM and rat NAALADase, pig FGCP lacks an N-terminal signal sequence but contains positively charged residues at the N-terminal side of the transmembrane domain that are characteristic of type II membrane proteins (25), while the remainder of the molecule containing the catalytic domain occupies an extracellular site. The putative catalytic domain of human PSM and rat NAALADase is conserved in FGCP between residues 275 and 588. Twelve NX(S/T) potential glycosylation sites occur at Asn positions 51, 77, 122, 141, 154, 196, 337, 460, 477, 614, 639, and 646, of which 10 are conserved by human PSM and nine by rat NAALADase. Five putative catalytic zinc binding residues are conserved at positions His^{378}, Asp^{388}, Glu^{426}, Asp^{456}, and His^{550}. Within the proposed specificity pocket, four positively charged residues are conserved at Arg^{464}, Lys^{561}, Arg^{525}, and Lys^{547}.

Homologies with Other Relevant Proteins—The BESTFIT computer program was used to analyze regional amino acid sequence homologies between pig FGCP and selected structurally and functionally related proteins (Table I). In addition to extensive sequence similarities and identities among FGCP, PSM, and NAALADase, FGCP exhibited similarities with three other M28 family members: human transferrin receptor (26) and aminopeptidases from *Vibrio proteolyticus* (27) and *Streptomyces griseus* (28). Rat 1100, a recently characterized ileal peptidase with type II structure (29), also shares extensive amino acid similarity with FGCP, whereas there was less sequence similarity between FGCP and human dipeptidyl peptidase IV, an enzyme that appears to be functionally related to 1100 (30). The PILEUP program was used to clarify amino acid alignments within the putative catalytic regions of FGCP, rat ileal 1100 (29), and human dipeptidyl peptidase IV (30). All five putative catalytic zinc binding residues (11) were conserved between pig jejunal FGCP and rat ileal 1100 at His^{378}, Asp^{388}, Glu^{426}, Asp^{456}, and His^{550}, while only one zinc binding residue at Glu^{426} was conserved in dipeptidyl peptidase IV. Among the putative substrate binding basic amino acids (11) that were conserved in FGCP, PSM, and NAALADase, only Arg^{464} was conserved in 1100, and only Arg^{537} was conserved in dipeptidyl peptidase IV. Several amino acids typical of a serine carboxypeptidase mechanism (29) were conserved further downstream, including Ser^{652} in all three proteins and Asp^{667} and His^{690} in FGCP and 1100. Structural similarities between FGCP and selected other proteins relevant to folate hydrolysis and transport were also investigated. Human glutamate hydrolase (an intracellular peptidase capable of folylpoly-γ-glutamate hydrolysis (31)) and two proteins involved in the transport of monoglutamyl folates (the mouse reduced folate carrier protein (RFC) (32) and pig folate-binding protein (FBP) (33)) showed only weak similarities to short regions at the N- or C-terminal ends outside of the catalytic region of FGCP.

Enzyme Activities—As depicted in Fig. 2, NAALADase-specific activity was 16-fold greater in pig jejunal brush-border membranes than in ileal brush-border membranes. NAALADase was abundant in membranes from PC3 cells transfected with the cDNA of pig jejunal FGCP but was absent from control PC3 cells. Previously characterized inhibitors (9, 20) nearly eliminated NAALADase activity in jejunal brush-border membranes and in FGCP transfectant membranes but had minimal effect on NAALADase activity in ileal brush-border membranes.

As depicted in Fig. 3 (*left panel*), FGCP activity in PC3 transfectant membranes was maximal at pH 6.5 and was not inhibited by the addition of *p*-hydroxymercuribenzoate to the reaction mixture. FGCP activity with an identical pH profile and lack of *p*-hydroxymercuribenzoate inhibition was found in PC3 cells transfected with the cDNA of PSM (not shown). By contrast, folate hydrolysis was much less in membranes of

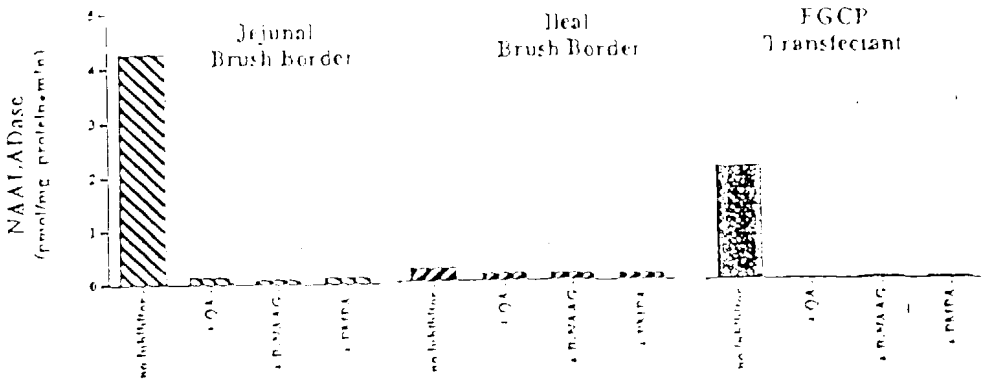

FIG. 2. NAALADase activity in pig jejunal and ileal brush-border membranes and in membranes of FGCP transfectants. Reaction mixtures included substrate NAAG (2.5 nM), jejunal brush-border membrane protein (2 μg), ileal brush-border membrane protein (20 μg), and FGCP transfectant membrane protein (2 μg), and NAAG inhibitors quisqualic acid (*QA*, 50 μM), β-*N*-acetylaspartylglutamate (β-*NAAG*, 25 μM), and 2-(phosphonomethyl)pentanedioic acid (*PMPA*, 10 nM). Data are expressed as the mean of three assays. Jejunal brush-border membranes demonstrated 16-fold greater NAAG-hydrolyzing activity than ileal brush-border membranes (4.275 ± 0.066 *versus* 0.253 ± 0.002 pmol/mg of protein•min). FGCP transfectants demonstrated NAAG-hydrolyzing activity (2.112 ± 0.073 pmol/mg of protein•min), while activity was negligible in controls (0.006 ± 0.010 pmol/mg protein•min). NAALADase inhibitors reduced NAAG hydrolysis to a greater extent in jejunal and FGCP transfectant membranes (>97% each) than in ileal membranes (44–48%).

mock transfected PC3 cells and exhibited a different optimal pH 4.5 with complete inhibition by *p*-hydroxymercuribenzoate. The kinetic characteristics of FGCP activity were compared in membranes from FGCP and PSM transfectants and in purified pig jejunal brush borders. As shown in Fig. 3 (*right panel*) and summarized in Table II, K_m and V_{max} values were similar in all three samples and were consistent with the kinetic profile of purified pig jejunal brush-border FGCP (4).

Immunoblots—Fig. 4 compares the immunoreactivities of the monoclonal antibody Mab-3 (5) with purified native pig FGCP, with pig FGCP transfectant membranes before and after treatment with peptide-*N*-deglycosidase F, and with human PSM transfectant membranes. Mab-3 detected the native pig FGCP and the pig FGCP transfectant glycoprotein at the identical size of 120 kDa and detected the deglycosylated polypeptide at 84 kDa but did not react with the human PSM transfectant membranes or with mock transfected control membranes.

Northern Blots—The cDNA of pig FGCP showed a strong hybridization signal at 2.8 kb in pig duodenum and jejunum and a faint signal in pig kidney, while no signal was detected in pig liver or ileum (Fig. 5). A band of similar size was identified in RNA extracts from pig and human jejunal mucosa. A positive actin signal was present in all samples. Several bands of hybridization appeared in RNA samples from rat and human brain and the LNCaP prostate carcinoma cell line (Fig. 6). Bands of roughly equal intensity were observed in rat brain at approximately 3.9, 2.95, and 2.8 kb, while a predominant species of 2.8 kb was found in human brain and in the human LNCaP prostate cancer cell line.

DISCUSSION

The present study has achieved the original molecular characterization of FGCP from pig jejunal mucosa. The authenticity of the pig FGCP cDNA sequence and its specific functional expression was established by (*a*) the incorporation of two native peptide sequences into the deduced amino acid sequence (Fig. 1), (*b*) the reproduction of the activity profile and kinetics of native pig FGCP (2, 4) in FGCP transfectant membranes (Fig. 3), (*c*) the immunoblot identification of the FGCP transcript by monoclonal antibody to native pig FGCP at the identical 120-kDa molecular size of the purified native enzyme (Ref. 5; Fig. 4) and identification of the deglycosylated polypeptide at the 84-kDa molecular size predicted by the amino acid sequence (Fig. 1), and (*d*) the identification of FGCP transcripts at 2.8 kb in pig jejunal mucosa and their absence in pig ileal mucosa (Fig. 5), consistent with the established intestinal distribution of the activity and immunoreactivity of the native enzyme (5). The additional presence of similar FGCP transcripts in pig and human jejunal mucosa (Fig. 5) suggests that the same gene expresses FGCP in human and pig jejunal brush-border membranes (2, 5).

The present experiments complete a circle of evidence for extensive molecular homologies among pig FGCP, human PSM, and rat NAALADase. The findings of 83–91% amino acid sequence identities between pig FGCP and each of the other sequences (Fig. 1; Table I) is in keeping with prior reports on the extensive amino acid identities between human PSM and rat NAALADase (6–9, 11) and is consistent with the concept that all three proteins represent species-specific homologues of the same gene. While the amino acid sequence of each protein predicts a polypeptide molecular size of 84 kDa (Fig. 1; Refs. 6–8), the presence of 12 glycosylation sites accounts for the greater 120-kDa molecular size of native (5) or transfectant FGCP (Fig. 4) compared with the reported molecular sizes of 100 kDa for PSM with 10 glycosylation sites (6) and of 94 kDa for NAALADase with nine glycosylation sites (7, 8, 34). While the epitope for our monoclonal antibody to native pig FGCP is unknown, incomplete amino acid sequence identities and differences in glycosylation between pig FGCP and human PSM could account for the lack of antibody cross-reactivity with PSM in transfectant membranes (Fig. 4). Prior findings of NAALADase transcripts at 2.8 kb in rat kidney (7, 8) are extended by the detection of a weak FGCP hybridization signal at 2.8 kb in pig kidney poly(A$^+$) RNA (Fig. 5), while the prior findings of PSM-like transcripts and immunoreactivity in human small intestine (35–37) are complemented by the detection of the FGCP hybridization signal at 2.8 kb in pig duodenal and jejunal poly(A$^+$) RNA and in human jejunal RNA (Fig. 5). The tissue distribution and predominant size of FGCP-like transcripts in rat and human brain and LNCaP cells (Fig. 6) is similar to other descriptions of the distribution and sizes of PSM and NAALADase transcripts in these tissues (6–9, 38). The previous finding of NAALADase activity in membranes of LNCaP cells and PSM transfectants (9) is complemented by finding NAALADase activity in pig jejunal brush-border membranes and in FGCP transfectant membranes (Fig. 2). The

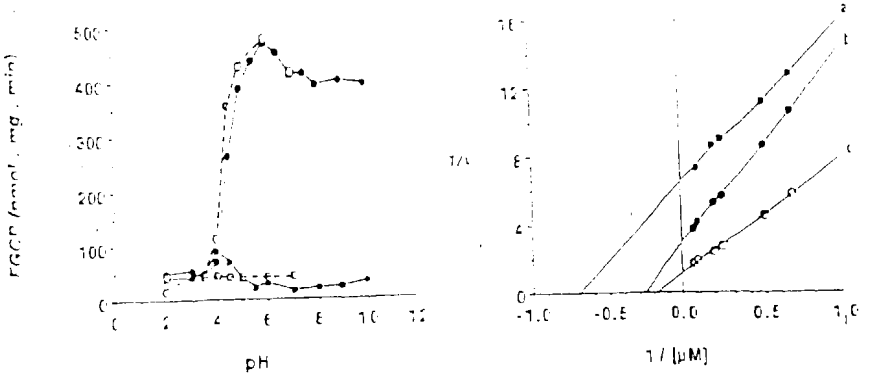

FIG. 3. Folate hydrolysis by membranes from native pig jejunal brush borders, mock transfected PC3 cells, and PC3 cells transfected with the cDNA of FGCP or PSM. Reaction mixtures consisted of 32 μM substrate folyl-γ-Glu-γ-[^{3}H]Glu in 55 mM 3,3-dimethylglutarate buffer containing 0.5 mM zinc acetate and 0.67 M NaCl in the final concentration. *Left panel*, Effect of varied buffer pH on folate hydrolysis by membranes from mock transfected and FGCP-transfected PC3 cells. FGCP activity was optimal in FGCP transfectant membranes at pH 6.0 (*closed circles*), in contrast to lesser folate hydrolysis in mock transfected PC3 cell membranes at optimal pH 4.0 (*closed boxes*). The addition of 0.5 mM *p*-hydroxymercuribenzoate in the final concentration had no effect on FGCP activity in FGCP transfectant membranes (*open circles*) but resulted in complete inhibition of folate hydrolysis in control PC3 cell membranes (*open boxes*). The FGCP activity profile of membranes of PSM transfectants was identical to that of FGCP transfectants (not shown). *Right panel*, kinetics of FGCP activity in membranes from pig jejunal brush borders and PC3 cells transfected with the cDNA of FGCP or PSM. Lineweaver-Burk plots of kinetics at pH 6.5 over a range of folyl-γ-Glu-γ-[^{3}H]Glu substrate concentrations show near identity among the membranes. *c*, PSM transfectant membranes; *b*, purified native pig jejunal brush-border membranes; *a*, FGCP transfectant membranes. K_m and V_{max} kinetic values are compared in Table II.

TABLE II

FGCP kinetics in native pig and transfectant cell membranes

A summary of activity constants (K_m) and maximal activities (V_{max}) of FGCP in membranes from purified pig jejunal brush borders, PC3 cells transfected with the cDNA of FGCP or PSM, and previously reported purified native pig jejunal FGCP (4). Kinetic data were obtained from studies that used a range of concentrations of substrate folyl-γ-Glu-γ-[^{3}H]Glu at pH 6.5 and conditions as described under "Experimental Procedures," followed by Lineweaver-Burk analysis of the results as shown in Fig. 5.

Source	K_m	V_{max}
	μM	nmol · mg · min
Pig jejunal brush border membrane	3.9	338
FGCP transfectant membrane	5.8	656
PSM transfectant membrane	1.4	152
Purified pig jejunal FGCP (4)	1.7	540

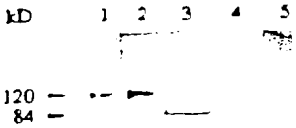

FIG. 4. Immunoblots showing the reaction of monoclonal antibody to native pig FGCP (5) to transfectant membrane proteins. Seven μg of solubilized membrane protein was added to each lane. An identical protein band was identified at 120 kDa in purified native pig FGCP (*lane 1*) and in membranes from the FGCP transfectant (*lane 2*), while the deglycosylated FGCP polypeptide appeared at 84 kDa (*lane 3*). Protein bands were absent from membranes of PSM transfectants (*lane 4*) and mock transfected PC3 cells (*lane 5*).

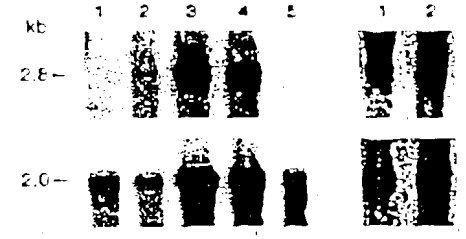

FIG. 5. Northern hybridization of ^{32}P-labeled pig FGCP cDNA and human β-actin to pig and human tissues. *Left panel*, a band of hybridization at 2.8 kb was prominent in poly(A$^+$) RNA from pig duodenal and jejunal mucosa (*lanes 3* and *4*), present in kidney (*lane 2*), and absent from liver (*lane 1*) and ileal mucosa (*lane 5*). *Right panel*, bands of hybridization of similar intensities were found at 2.8 kb in total RNA from pig (*lane 1*) and human jejunal mucosa (*lane 2*). Control hybridization to actin is shown at 2.0 kb.

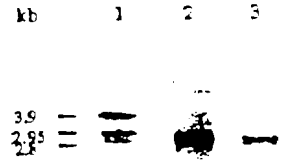

FIG. 6. Northern hybridization of ^{32}P-labeled pig FGCP cDNA to brain and prostate RNAs. Samples contained different amounts of total RNA in rat brain (10 mg) and LNCaP cells (5 mg) and poly(A$^+$) RNA in human brain (2 mg). A longer exposure was required to develop the signal from rat brain. Bands of hybridization were observed in rat brain RNA at 3.9, 2.95, and 2.8 kb (*lane 1*). A predominant hybridization signal appeared at 2.8 kb in LNCaP cell RNA (*lane 2*) and in human brain poly(A$^+$) RNA (*lane 3*).

observation that membranes of LNCaP cells or PSM transfectants were capable of progressive hydrolysis of folylpoly-γ-glutamates (10) is confirmed and extended by finding nearly identical kinetic properties of purified native FGCP in FGCP or PSM transfectant membranes (Fig. 3; Table II).

A recent analysis classified human prostate PSM and rat brain NAALADase as GCP II, a single type II glycoprotein member of the M28 family of peptidases (11) (EC 3.4.17.21). The extensive amino acid identities, common structural motifs, and conservation of the identical five co-catalytic zinc-binding amino acids and four putative substrate binding basic amino acids suggest that FGCP derives from the pig homologue of the GCP II gene (Fig. 1). GCP II and two prototypical bacterial aminopeptidases *V. proteolyticus* (27) and *S. griseus* (28) are members of the M28 peptidase family by virtue of homologous catalytic domains, which appear to bind two co-catalytic zinc

atoms (31, 39). The three-dimensional structural analysis of *V. proteolyticus* aminopeptidase suggested the location of a substrate specificity pocket, which is composed of basic amino acids in PSM and NAALAdase (11, 27). The loci of the human PSM gene and a second similar sequence have been found on human chromosome 11 (40, 41). Others recently identified another type II ileal brush-border membrane protein, J100, that shares 60 and 59% sequence identities with rat NAALAdase and human PSM (29), of which the human homologue might comprise the second locus on chromosome 11. J100 exhibits activity similar to human dipeptidyl peptidase IV, another peptidase associated with the apical brush border of intestinal epithelial cells (29, 30). These relationships prompted our evaluation of potential structural similarities among FGCP, J100, and dipeptidyl peptidase IV. The conservation of all five zinc-binding residues suggests that FGCP and J100 share the same catalytic mechanism. On the other hand, an alternative potential serine carboxypeptidase mechanism (29) is suggested by conservation of Ser^{636} in all three sequences.

While pig FGCP, rat NAALAdase, and human PSM may represent different species-specific expressions of same GCP II gene, their functions appear to differ according to the tissue in which the gene is expressed. Thus, GCP II may function as FGCP in the jejunum by cleaving γ-linked glutamyl residues sequentially from dietary folylpoly-γ-glutamates prior to the intestinal transport of folic acid (1, 2, 4, 5) and as NAALAdase in the brain to release α-linked glutamate from NAAG to regulate subsequent neurotransmission (8, 9). These different functions may reflect tissue differences in available substrate, since NAAG is concentrated at neuronal synapses (8), while folylpoly-γ-glutamates are concentrated as dietary components at the brush-border surface of the proximal small intestine (1).

The present study offers molecular clarity to the mechanism of folate absorption at the intestinal brush-border membrane. Our original studies identified an initial stage of jejunal hydrolysis of dietary folylpoly-γ-glutamates that precedes the intestinal uptake of the folic acid product (1). We identified and characterized FGCP as a zinc-dependent exopeptidase that is active at a neutral pH optimum in human and pig jejunal brush-border membrane fractions (2, 4) and that was localized in the pig to the jejunal brush-border membrane and was excluded from the ileal brush-border membrane by the monoclonal antibody Mab-3 to the purified enzyme (5). These observations are extended by the present molecular characterization of FGCP as a type II protein of the M28 peptidase family with a zinc-binding motif, for which the transcripts are expressed in proximal but not distal pig small intestine (Fig. 5). The finding of a different activity profile of folate hydrolysis by mock transfected PC3 cells including an acid pH optimum and complete *p*-hydroxymercuribenzoate inhibition (Fig. 3) is consistent with our prior definition of the characteristics of a separate lysosomal endopeptidase that provides intracellular folate hydrolysis in human and pig jejunal mucosa (3, 4). The recently described PSM′ splice variant (42) cannot provide the separate profile of folate hydrolysis found in mock transfected PC3 cells (Fig. 3), since no genetically similar species is expressed in native PC3 cells (6, 9). Alternatively, the second folate hydrolyzing activity in mock translated PC3 cell membranes (Fig. 3) and in the lysosomal fraction of jejunal mucosa (3) may be attributed to the recently described and genetically dissimilar glutamate hydrolase (EC 3.4.19.9) (Table I; Ref. 31).

The present studies provide a molecular framework for future studies on the regulation of FGCP by conditions known to affect intestinal folate absorption and on the relationship of FGCP to RFC and FBP, two proteins involved in membrane transport of monoglutamyl folates (Table I). The cDNA sequences of mouse and human RFC have been defined, and its intestinal transcription and functional capability for transport of monoglutamyl folate in cell transfectants has been proven (32, 43, 44). The alternate receptor FBP has been characterized at the molecular level in pig liver, but its transcripts and activity are absent from the jejunum (33).[3] The present study shows that FGCP is genetically distinct from both RFC and FBP, since their amino acid sequences are minimally represented in FGCP (Table I). In summary, the available data indicate that the intestinal absorption of dietary folylpoly-γ-glutamates is achieved by a two-step process of progressive hydrolysis of γ-linked glutamyl residues by FGCP at the jejunal brush-border membrane, releasing folic acid and other monoglutamyl folate derivatives for subsequent membrane transport by genetically distinct RFC. The integration of folate hydrolysis by jejunal FGCP and folic acid transport by intestinal RFC in the overall process of folate absorption has yet to be defined. These studies are now feasible due to the molecular identification of FGCP.

REFERENCES

1. Halsted, C. H. (1990) in *Folic Acid Metabolism in Health and Disease* (Picciano, M. F., Stokstad, E. L. R., and Gregory, J. F., III) pp. 23–45, Wiley-Liss, New York
2. Chandler, C. J., Wang, T. T. Y., and Halsted, C. H. (1986) *J. Biol. Chem.* 261, 928–933
3. Wang, T. T. Y., Chandler, C. J., and Halsted, C. H. (1986) *J. Biol. Chem.* 261, 13553–13558
4. Chandler, C. J., Wang, T. T. Y., and Halsted, C. H. (1986) in *Chemistry and Biology of Pteridines* (Cooper, B. A., and Whitehead, V. M.) pp. 539–542, Walter de Gruyter & Co., New York
5. Chandler, C. J., Harrison, D. A., Buffington, C. A., Santiago, N. A., and Halsted, C. H. (1991) *Am. J. Physiol.* 260, G665–G672
6. Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. W. (1993) *Cancer Res.* 53, 227–230
7. Bzdega, T., Turi, T., Wroblewska, B., She, D., Chung, H. S., Kim, H., and Neale, J. H. (1997) *J. Neurochem.* 69, 2270–2277
8. Luthi-Carter, R., Berger, U. V., Barczak, A. K., Ennis, M., and Coyle, J. T. (1998) *Proc. Natl. Acad. Sci.* 95, 3215–3220
9. Carter, R. E., Feldman, A. R., and Coyle, J. T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 749–753
10. Pinto, J. T., Suffoletta, E. P., Berzin, T. M., Qiao, C. H., Lin, S., Tong, W. P., May, F., Mukherjee, B., and Heston, W. D. W. (1996) *Clin. Cancer Res.* 2, 1445–1451
11. Rawlings, N. D., and Barrett, A. J. (1995) *Biochim. Biophys. Acta* 1339, 247–252
12. Matsudaira, P. (1989) *A Practical Guide to Protein and Peptide Purification for Microsequencing*, Academic Press, Inc., San Diego
13. Chirgwin, J. J., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* 18, 5294–5299
14. Wang, A. M., Doyle, M. V., and Mark, D. F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 9717–9721
15. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
16. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 69, 1408–1412
17. Benton, W. D., and Davis, R. W. (1977) *Science* 196, 180–182
18. Frohman, M., Dush, M., and Martin, G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 8998–9002
19. Graham, F. L., and van der Eb, A. J. (1973) *Virology* 52, 456–467
20. Robinson, M. E., Blakely, R. D., Couto, R., and Coyle, J. T. (1987) *J. Biol. Chem.* 262, 14498–14506
21. Krumdieck, C. L., and Baugh, C. M. (1970) *Anal. Biochem.* 36, 123–129
22. Laemmli, U. K. (1970) *Nature* 227, 680–685
23. Sambrook J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* 157, 105–132
25. Parks, G. D., and Lamb, R. A. (1991) *Cell* 64, 777–787
26. McClelland, A., Kuhn, L. C., and Ruddle, F. H. (1984) *Cell* 39, 267–274
27. Chevrier, B., D'Orchymont, H., Schalk, C., Tarnus, C., and Moras, D. (1996) *Eur. J. Biochem.* 237, 393–398
28. Matias, B., Greenblatt, H. M., Shoham, G., Spungin-Bialik, A., Blumberg, S., and Barra, D. (1996) *Eur. J. Biochem.* 236, 845–846
29. Schneider, B. L., Thevananther, S., Moyer, M. S., Walters, H. C., Rinaldo, F., Deevarajan, P., Sun, A. Q., Dawson, P. A., and Ananthanarayanan, M. (1997) *J. Biol. Chem.* 272, 31006–31013
30. Darmoul, D., Lacasa, M., Baricault, L., Marguet, D., Sapin, C., Trotot, P., Barbot, A., and Trugnan, G. (1992) *J. Biol. Chem.* 267, 4824–4833
31. Yoo, E., Schneider, E., Ryan, T. J., and Gelfman, J. (1996). *Proc. Soc. Natl. Acad. Sci. U. S. A.* 93, 10134–10138
32. Dixon, K. H., Lanpher, B. C., Chiu, J., Kelley, K., and Cowan, K. H. (1994)

[3] J. A. Villanueva and C. H. Halsted, unpublished data.

J. Biol. Chem. 268, 15–20
35. Van Heuzen, C. M., Linn, E.-H., and Halsted, C. H. (1996) *Biochem. J.* 315, 725–729
36. Slusher, B. S., Robinson, M. B., Tsai, G., Simmons, M., Richards, S. S., and Coyle, J. T. (1992) *J. Comp. Neurol.* 318, 217–225
37. Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R., and Heston, W. D. W. (1995) *Cancer Res.* 64, 1807–1811
38. Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D. W., and Cordon-Cardo, C. (1997) *Clin. Cancer Res.* 3, 81–85
39. Troyer, J. K., Beckett, M. L., and Wright, G. L. (1995) *Int. J. Cancer* 62, 552–558
40. Luthi-Carter, R., Barczak, A. K., Speno, H., and Coyle, J. T. (1998) *J. Pharmacol. Exp. Ther.* 286, 1020–1028
41. Valicenti, B. L., and Auld, D. (1993) *Proc. Soc. Natl. Acad. Sci. U. S. A.* 90, 2715–2718
42. Rinker-Schaeffer, C. W., Hawkins, A. L., Su, S. L., Israeli, R., Griffin, C. A., Isaacs, J. T., and Heston, W. D. W. (1995) *Genomics* 30, 105–108
43. Leek, J., Lench, N., Merry, E., Bailey, A., Carr, I. M., Andersen, S., Crowe, J., Whelan, P., MacLennan, K. A., Meredith, D. M., and Markham, A. F. (1996) *Br. J. Cancer* 73, 585–588
44. Su, S. L., Huang, I.-P., Fair, W. R., Powell, C. T., and Heston, W. D. W. (1995) *Cancer Res.* 55, 1441–1443
45. Said, H. M., Nguyen, T. T., Dyer, D. L., Cowan, K. H., Rubin, S. A. (1990) *Biochim. Biophys. Acta* 1281, 164–172
46. Nguyen, T. T., Dyer, D. L., Dunning, D. D., Rubin, S. A., Grant, K. E., Said, H. M. (1997) *Gastroenterology* 112, 783–791